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EXHIBIT I

Characterization of Potential Insertion Sites in the Core Antigen of Hepatitis B Virus by the Use of a Short-Sized Model Epitope[1]

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Summary
Core particles of hepatitis B virus (HBV) are able to
improve the immunogenicity of foreign sequences ex-
posed on the particle surface. The insertion site in the
core antigen of HBV (HBcAg) determines the surface pre-
sentation and thus the immunogenicity of the foreign
sequence. For direct comparison of the value of potential
insertion sites in the core antigen, we constructed vec-
tors allowing insertions of a model marker epitope
DPAFR. This epitope was inserted at the N-terminus, the
c/e1 loop, behind amino acid (aa) 144 and behind aa 183
(DPAF only). In addition, we generated a mosaic con-
struct allowing the co-expression of HBcAg and a HBcAg/
DPAFR fusion protein due to a suppressor tRNA-me-
diated readthrough mechanism. All 6 constructs allowed
the formation of chimaeric or mosaic core-like particles.
Western blot analyses and a direct ELISA demonstrated
the presence of the DPAFR sequence in the chimaeric
and mosaic particles. Competitive ELISA and immune

electron-microscopic data suggested the c/e1 loop as the
insertion site of choice for presenting foreign sequences
on the surface of chimaeric HBV core particles. However,
the N-terminal fusion also allowed partial surface expo-
sure of the DPAFR motif. In contrast, in particles of con-
structs carrying the DPAFR insert at aa position 144 or
183, respectively, the epitope seemed not to be surface
accessible.

Virus-like particles generated by heterologous expres-
sion of viral structural proteins can be used as vaccines or
diagnostic antigens. In recent years, core particles of hepa-
titis B virus (HBV) obtained by the expression of the core
antigen of HBV (HBcAg) in *Escherichia coli* cells became
useful carrier molecules for foreign epitopes due to their
favourable immunogenic properties [for a review, see
ref.1]. Cryo-electron microscopy data [2] and epitope
mapping data of HBV core [3, 4] suggested the so-called
c/e1 loop region located around amino acid (aa) position
80 in HBcAg to be a preferable insertion site for foreign
sequences. However, various reports demonstrated the
possibility to fuse foreign sequences also N- or C-termi-
nally to the HBcAg [for a review, see ref. 1].
    Investigations of various insertion sites in HBcAg by
the use of different epitopes revealed the c/e1 loop inser-

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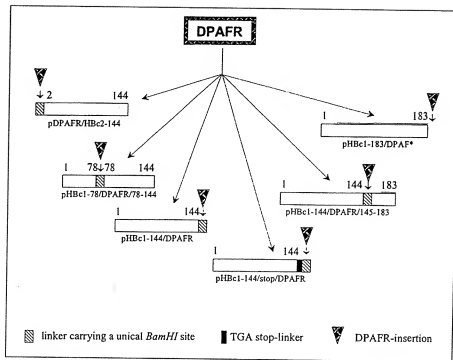Tel. +49 30 2802 2387, Fax +49 30 2802 2180

Fig. 1. Primary structure of plasmids encoding HBcAg/DPAFR fusion proteins. Numbers indicate aa position in HBcAg. * Construct contains the tetrapeptide DPAF [9].

⬚ linker carrying a unical *BamHI* site   ▮ TGA stop-linker   ▼ DPAFR-insertion

tions to be the best in respect of both antigenicity and immunogenicity [5–8]. However, a systematic study of potential insertion sites in HBcAg by one defined epitope is still missing. For this purpose, we made use of a short-sized DPAFR epitope of HBV preS1 [4, 9] being recognized by the monoclonal antibody MA18/7 [10]. Here we present a comparative analysis of particle formation, antigenicity and surface accessibility of the DPAFR epitope inserted into different sites of HBcAg. In our study, we used the 183-aa-long HBcAg or a truncated form of 144 aa named HBcAgΔ.

For evaluation of different insertion sites in terms of presentation of foreign epitopes on the surface of the core-like particles, vectors were constructed allowing fusions to the N-terminus as well as insertions at the c/e1 loop and at various sites in the C-terminal region of HBcAg (fig. 1). The generation of the trp promoter-carrying plasmids pHBc1–78/DPAFR/78–144 (insertion of DPAFR at aa position 78 of a C-terminally truncated HBcAg) and pHBc1–183/DPAF (fusion of DPAF behind aa position 183 of the entire HBcAg) have already been described [9, 11;· original plasmid designations are pHBc2–19 and pHBcAg-DPAFR183, respectively]. New vectors were generated in order to insert foreign sequences into a unique *Bam*HI site with a GAT (Asp) reading frame at the above-mentioned positions. The plasmid pN1HBc2–144

(basis of pDPAFR/HBc2–144) carries a phage T5 promoter/lac operator whereas all other plasmids contain a trp promoter: pHBc2-9FS (basis of pHBc1–144/DPAFR), pHBc2-9FS-16¹⁵ (basis of pHBc1–144/DPAFR/145–183) and pHBc2-9FS-stop (basis of pHBc1–144/stop/DPAFR) [12].

The above-mentioned vectors were linearized by *Bam*-HI cleavage and dephosphorylated. Into the *Bam*HI site an oligonucleotide duplex was inserted generated by hybridization of the two following oligonucleotides:

    5′-GAT CCG GCT TTC CGT AGG CCT-3′
       3′-GC CGA AAG GCA TCC GGA CTA G-5′
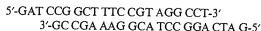
After transformation, *E. coli* K12 XL-1 Blue cells were screened for recombinant plasmids. Recombinants carrying a single copy of the DPAFR insert in the correct orientation were identified by DNA sequence analysis.

For expression of the HBcAg-derived fusion proteins, the plasmids pHBc1–78/DPAFR/78–144, pHBc1–183/DPAF, pHBc1–144/DPAFR, pHBc1–144/DPAFR/145–183 were re-transformed into *E. coli* K12 K802 cells. In the case of the mosaic construct pHBc1–144/stop/DPAFR an *E. coli* K12 K802 strain carrying the plasmid pISM 3001 [13] was used to allow UGA stop codon suppression. The plasmid pDPAFR/HBc2–144 was re-transformed

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into M15pREP4 cells. The trp promotor- or T5 promoter/Lac operator-driven expression of the HBcAg fusion proteins was induced by addition of indole acrylic acid and IPTG, respectively, resulting in the synthesis of HBcAg fusion proteins of the predicted molecular weights (fig. 2). In the lysate of pHBc1–144/stop/DPAFR-transformed suppressor cells (lane E) a HBcAgΔ (MW 16.9 kD) and a HBcAgΔ/DPAFR readthrough protein (MW 19.8 kD) were identified. Only the HBcAg fusion protein expressed by pHBc1–183/DPAF migrated slightly slower than predicted (lane H; predicted MW 22.3 kD). In cells expressing proteins with the DPAFR insert at the c/e1 or C-terminal site (but not at the N terminus) a smaller immunoreactive protein band was observed (fig. 2, c; lanes D–H). Since these proteins do react with the monoclonal antibody MA18/7 (fig. 2c), they should represent an N-terminal degradation product.

To assess core particle formation and their antigenicitiy, approximately 0.5–1.0 g of induced *E. coli* cells were lysed by lysozyme treatment, three cycles of freezing and thawing and sonication. Soluble proteins were precipitated by addition of ammonium sulphate (final concentration 30% at 4°) and purified by ultracentrifugation at 28,000 rpm for 20 h at 4° (Beckman, L7-55, rotor SW28) in a discontinuous 20–50% sucrose gradient. The peak HBc/HBe antigenicity analysed by Western blot was found in fraction 5 or 6 corresponding to a buoyant density of 1.16–1.17 g/cm³ for both HBcAgΔ (aa 1–144) itself and HBcAgΔ fusion proteins. The constructs pHBc1–183/DPAF and pHBc1–144/DPAFR/145–183, however, carrying the C-terminal arginine-rich part of HBcAg were found at a higher density (fraction 3 corresponding to 1.19 g/cm³). The HBcAg-derived fusion proteins reacted in Western blots both with an anti-HBc/HBe rabbit serum (fig. 2b) and with the monoclonal antibody MA18/7 directed against the DPAFR motif (fig. 2c). The fusion proteins carrying aa 145–183 of HBcAg were also detected by an anti-peptide serum (data not shown) raised against aa 173–182 of the HBcAg C terminus [14]. Indications for the potential of the HBcAg-derived proteins to form particles were obtained by radial immunodiffusion analysis using a human HBc-specific serum (data not shown) and by an HBc-specific sandwich ELISA (tables 1 and 2; for details see legend of table 1). The formation of core-like particles was verified by negative-staining electron microscopy (fig. 3).

The presence of the DPAFR peptide in particles of all constructs was confirmed by a direct ELISA whereas the surface exposure of DPAFR was indicated by a sandwich ELISA specific for core particles due to recognition of the
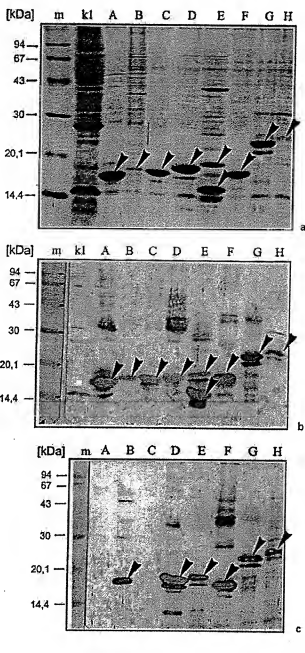

Fig. 2. Detection of HBcAg-derived proteins in Coomassie blue stained 15% SDS-PAGE (**a**) and Western blots using a HBc/HBe-specific rabbit serum (**b**) or anti-preS1 antibody MA 18/7 (**c**). Purified particles from sucrose gradient fractions were applied on the gel: pN1HBc2–144 (A); pDPAFR/HBc2–144 (B); pHBc2–9FS (C); pHBc1–144/DPAFR (D); pHBc1–144/stop/DPAFR (E); pHBc1–78/DPAFR/78–144 (F); pHBc1–144/DPAFR/145–183 (G); pHBc1–183/DPAF (H). Lysate of pQE40-transformed M15pREP4 cells (lane k1) was applied as control. Lane m: molecular weight marker proteins phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), α-lactalbumin (14.4 kD).

Table 1. Analysis of the HBc antigenicity and the presence of the DPAFR sequence

Expression product	Immunodiffusion endpoint titer	HBc antigenicity sandwich ELISA[a] absorbance	DPAFR antigenicity direct ELISA[b] absorbance	sandwich ELISA[c] absorbance
pN1HBc2–144	1:16	0.379 (10^{-6})	0.025	0.03
pDPAFR/HBc2–144	–	0.493 (10^{-2})	1.064	0.625
pHBc1–78/DPAFR/78–144	1:16	0.165 (10^{-6})	2.393	1.577
pHBc2–9FS	1:16	0.133 (10^{-6})	0.002	0.028
pHBc1–144/DPAFR	1:16	0.268 (10^{-6})	0.92	0.018
pHBc1–144/stop/DPAFR	1:16	0.993 (10^{-6})	0.187	0.042
pHBc1–144/DPAFR/145–183	1:16	0.207 (10^{-5})	0.173	0.04
pHBc1–183/DPAF	1:16	0.501 (10^{-3})	0.164	0.045

Absorption was measured at 450 nm (reference 620 nm). The data represent single ELISA investigations.

[a] Plates were coated with anti-human-HBc-IgG followed by incubation of dilutions of the purified core particles. Peroxidase-labeled human anti-HBc-IgG was used for detection. The dilution of the sucrose gradient fractions is given in parentheses.

[b] 0.1 μg of the purified particles were adsorbed to the plates and DPAFR was detected using MA18/7.

[c] Again plates were coated with anti-HBc-IgG and incubated with 1:10 diluted sucrose gradient fractions. The epitope exposure of the core particles was measured using MA18/7.

Table 2. Antigenicity of the chimaeric and mosaic core particles

Designation	HBc antigenicity[a]	DPAFR antigenicity[b]	DPAFR exposure[c]
pDPAFR/HBc2–144	+	++	+
pHBc1–78/DPAFR/78–144	+++	+++	+++
pHBc1–144/DPAFR	+++	++	–
pHBc1–144/stop/DPAFR	+++	+	–
pHBc1–144/DPAFR/145–183	+++	+	–
pHBc1–183/DPAF	+	+	–

[a] Compare table 1 for detailed results. + = low antigenicity (HBc ELISA positive at a dilution of $10^{-2}/10^{-3}$; no reactivity in immunodiffusion); +++ = high antigenicity (HBc ELISA positive at a dilution of $10^{-5}/10^{-6}$; reactivity in immunodiffusion).

[b] DPAFR antigenicity was demonstrated using a direct ELISA (see table 1). + = OD 0.1–0.2; ++ = OD 0.9–1.0; +++ = OD >2. Similar results were obtained by Western blot analysis (see fig. 2).

[c] A sandwich ELISA (table 1), a competitive ELISA and immune electron microscopy (fig. 3) were used to verify exposure of the epitope. The data of all three assays are in accordance. The competitive ELISA was carried out as follows: the plates were coated with 0.1 μg of particles expressed by pHBc1–78/DPAFR/78–144. In the next step, MA18/7 as well as increasing concentrations of the peak sucrose gradient fractions of each construct were added at the same time. The competition between particles adsorbed to the plates and particles in suspension for binding of MA18/7 was measured in a staining reaction at 450 nm (reference 620 nm). Therefore, peroxidase-labeled anti-mouse IgG was used for detection. – = no competition; + = weak competition (in the case of pre-incubation of mab MA18/7 and core-like particles only); +++ = strong competition even without pre-incubation.

conformational 'c' epitope by the catching antibody (tables 1, 2). The latter assay revealed surface exposure of the DPAFR sequence only in the N-terminal and c/e1 loop position. These data were verified by a third assay format: a competitive ELISA (for details see legend of table 2) demonstrated a strong surface exposure of the c/e1-positioned epitope (data now shown). Only after allowing preincubation of the particles with MA18/7 was a significant but weaker accessibility for the N-terminally fused DPAFR observed (table 2). These data were corroborated by immune electron microscopy (fig. 3) were in case of the constructs pHBc1–78/DPAFR/78–144 and pDPAFR/HBc2–144 was a surface exposure of the DPAFR sequence detected by binding of monoclonal antibody MA18/7, while the constructs carrying the epitope at positions 144 or 183 of HBcAg remained free from antibody tagging.

The data presented here are in line with the recently published fine structure of the HBcAg molecule demonstrating the c/e1 region as a protruding spike between aa 78–82 [2]. Experimental data on the insertion of foreign sequences into c/e1 confirmed this region as a preferential insertion site for the surface exposure and high immunogenicity of foreign sequences [for a review, see ref. 1]. Although fusions of the N terminus of HBcAg are not as antigenic and immunogenic as insertions into the c/e1 region [5, 6 and data presented here], the N-terminal insertion site could be of relevance for packaging of pro-

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Fig. 3. HBV-core-like particles with DPAFR insertion evaluated by direct immune electron microscopy using unlabelled antibody. Purified particles isolated from the sucrose gradient (or in the case of **b** and **g** from pooled sucrose fractions) were adsorbed with MA18/7 at a concentration of 0.05 or 0.2 mg/ml and incubated with MA18/7 at a concentration of 0.05 or 0.2 mg/ml and negative stained using 1% uranyl acetate. Whereas particles of pN1HBc2–144 without DPAFR insertion (**a**), pHBc1–144/DPAFR (**d**), pHBc1-144/stop/DPAFR (**e**), pHBc1–144/DPAFR/145–183 (**f**) and pHBc1-183/DPAF (**g**) showed no reaction with MA18/7, core-like particles of pHBc1–78/DPAFR/78–144 (**c**) were strongly decorated by a fringe of antibody and partly also immunoaggregated. Particles of pDPAFR/HBc2–144 (**b**) were recognized by MA 18/7 only after 1 h incubation of a 1:1 mixture of particles and antibody before applying the suspension onto the grid. Incubation (in suspension) of a yellow fever virus-specific monoclonal antibody with particles of pHBc1–78/DPAFR/78–144 serving as a negative control (**h**) did not result in immune-decoration. Bar represents 100 nm. × 80,000.
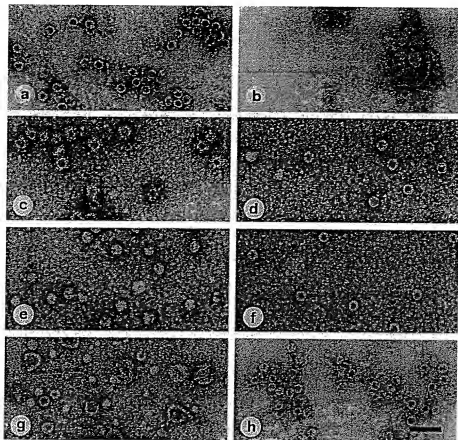
longed foreign sequences not tolerated in the c/e1 insertion site. Insertion of a spacer/linker, as a penta-glycine sequence (our data) or a pre-core sequence [6], between the foreign sequence and HBcAg could probably improve the surface exposure of N-terminal fused epitopes. However, other authors did not confirm the necessity of a linker insertion for surface exposure [15]. Similarly, C-terminal fusions were found again to be lower immunogenic than c/e1 insertions [8]. These data are in line with structural data suggesting a luminal localization of the C-terminal region [16]. In contrast to a previous report on the surface accessibility of the DPAFR epitope inserted behind aa 144 [4] we were unable to observe a surface exposure of the sequence at this site. These differences are probably due to additional amino acids located behind the DPAFR motif because of a longer preS1 insert used by Pushko et al. [4]. Other authors also discussed a potential spacer effect on the surface accessibility of a foreign sequence inserted behind aa position 149 [17]. Interestingly, in chimaeric core particles carrying 90 aa of HIV-1 Gag behind aa position 144 of HBcAgΔ, surface-exposed as well as non-exposed regions were mapped [18]. In line with data

of Yon et al. [7] who did insert an SIV epitope at this position we found the proximal C-terminal insertion site at aa position 183 not to be surface accessible.

In conclusion, our data presented the first comparison of various HBcAg insertion sites with regard to the surface accessibility of one and the same foreign sequence. Whether these findings can be extended to other foreign sequences will be proven in further investigations using the major protective region of a Puumala hantavirus nucleocapsid protein.

Acknowledgments

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